Investigation of *Avian polyomavirus* and *Psittacine beak* and *Feather disease virus* in parrots in Taiwan

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Abstract

This study aimed to understand the related statistics of APV and PBFDV in parrots, such as the positive percentage and the distributions of various ages, seasons, breeds and areas, in order to obtain more information on the health care of captive parrots in Taiwan today. Blood samples were collected from parrots that were household pets and from commercial aviaries in the northern (e.g. Taipei), central (e.g. Taichung) and southern (e.g. Kaohsiung) Taiwan regions randomly. Collection targets were mainly small and medium species such as *Psittacula krameri* and others such as *Psittacus erithacus*. Sixty samples were collected every month and a total of 720 samples in a year. DNA was extracted and analyzed by polymerase chain reaction (PCR), sequencing and statistical analysis. The results showed that 28.3% (204/720) were PBFDV positive, 8.6% (62/720) were APV positive in this study and they were different from the two previous studies of Taiwan and studies of other countries such as the USA and Italy; however, APV/PBFDV co-infection (12%) was similar to the two previous studies of Taiwan (10.3% and 11.04%). The main species and age most susceptible were *Psittacula krameri* (14.1%, 48.7%), Lorikeets (9.8%, 19.5%), *Aratinga solstitialis* (7.5%, 28%), *Eclectus roratus* (6.3%, 22.9%) and chicks that were under six-months-old (3.6%, 17.8%). APV and PBFDV are easily spread during the breeding season due to frequent contact which makes the proportion of positives higher from commercial aviaries (8.7%, 29.7%) than that from household pets (8.3%, 21.7%), mainly in spring (13.3%, 44.4%) and fall (13.9%, 38.9%).

Introduction

Avian polyomavirus (APV) is a 4981bp double-stranded circular DNA virus of the family *Polyomaviridae*, genus *Polyomavirus*; with non-enveloped, icosahedral symmetry, about 20-50 nm in diameter and 40-55 nm of Virion size (Alley *et al.*, 2013, Arroube *et al.*, 2009, Rott *et al.*, 1988). *Psittacine Beak and Feather Disease Virus* (PBFDV) is a 1993bp single-stranded circular DNA virus of the family *Circoviridae*, genus *Circovirus*; the virion is about 14-26 nm, with non-enveloped and icosahedral symmetry (Harkins *et al.*, 2014). APV and PBFDV are common feather shedding and fatal diseases in parrots which cause acute or chronic infection. The severity and sensitivity in parrots depend on the species and age of the parrot (Johne and Muller, 2007, Sanada *et al.*, 1999, Enders *et al.*, 1997, Crawford, 1994, Ritchie *et al.*, 1992, Latimer *et al.*, 1991).

APV has now been reported in the United States, Canada, Japan, Africa, Australia, central Europe and other countries. The case fatality rate in parrot chicks is 20% to 100%. The first case in Taiwan was in 1987 (Johne and Muller, 2007, Enders *et al.*, 1997, Phalen *et al.*, 1991, Gough, 1989, Hirai *et al.*, 1984, McOrist *et al.*, 1984, Pass and Perry, 1984, Dykstra and Bozeman, 1982, Bernier et al 1981) with a mortality rate of nearly 100% in budgerigars (Krautwald *et al.*, 1989, Kaleta *et al.*, 1984). In the case of non-budgerigar psittacine birds, except for chicks that are at higher risk, the mortality rate is about 27% to 41%, which is usually an inapparent infection that causes them to become carriers. (Graham and Calnek, 1987, Jacobson *et al.*, 1984). The virus can be detected in all organs with a wide range of multiple organ infection capabilities (Phalen *et al.*, 1993).

When APV infection is widespread all over the world, analysis shows that almost all infected cases have only one genotype and one serotype (Bert *et al.*, 2005, Hsu *et al.*, 2006, Khan *et al.*, 2000, Phalen *et al.*, 1999). Parrot species are more commonly infected with APV such as *Melopsittacus undulatus*, *Eclectus roratus*, Conures (genus *Aratinga/Pyrrhura*) and *Psittacula krameri*; while *Psittacus erithacus*, *Deroptyus accipitrinus* and Amazon parrots have fewer cases and APV also has a record of infection in non-parrot birds, such as *Pteroglossus viridis*, *Pyrenestes sanguineus*, *Chloris chloris* and *Accipitriformes* (vultures, falcons) (Johne and Muller, 2007, Rossi *et al.*, 2005, Lafferty *et al.*, 1999, Garcia *et al.*, 1994, Stoll *et al.*, 1993, Forshaw *et al.*, 1988).

PBFDV was first found in wild parrots in Australia. Due to the popularity of the parrot trade, transmission to the United Kingdom, Germany, Portugal, the United States, Spain, Israel, New Zealand, Zimbabwe and South Africa has been reported (Heath *et al.*, 2004, McOrist *et al.*, 1984, Pass and Perry, 1984). Acute infection by PBFDV occurs in parrots under three years old, usually at the first feather growth (about 28-32 days old) and some cause beak deformity (Sanada *et al.*, 1999, Crawford, 1994, Cooper *et al.*, 1987, McOrist *et al.*, 1984, Pass and Perry, 1984).

PBFDV affects over 40 different psittacine species (Gerlach, 1994). According to the literature published in 1990, the clinical and pathological differences in parrot species that are known to be more susceptible to PBFDV are only due to host factors rather than the differences in viral antigens and genes (or available variability) (Ritchie *et al.*, 1990). However, this was not been confirmed until 1993 when Raidal *et al.*, found that different virus strains can express the same antigen in blood clotting and blood clotting inhibition experiments (Raidal *et al.*, 1993).

Primers have been designed to amplify a fragment of the capsid protein region encoded by the ORF V1 gene of PBFDV genome to detect PBFDV in psittacine birds (Ypelaar *et al.*, 1999). Remarkable differences have been found between mammalian polyomaviruses and APV especially in the non-coding regulatory region and in the regions encoding the large tumor (T) antigen; therefore amplification of t/T antigen region of APV genome has been used and shown conservation in all APV-positive samples as expected (Dolz *et al.*, 2013, Ogawa *et al.*, 2005).

Reported viral DNA positive rates in different countries vary for PBFDV from 3.5% in the USA, 8.05% in Italy, 8.59% in Taiwan and 19.7% in Costa Rica to 40.4% in Germany and 41.2% in Taiwan, and for APV, from 0.79% in Italy and 4.8% in Costa Rica to 15.2% and 16.56% in Taiwan. Meanwhile, APV/PBFDV co-infections were 3.3% in Costa Rica to 10.3% and 11.04% in Taiwan (Thongchan *et al.*, 2015, Dolz *et al.*, 2013, Hsu *et al.*, 2006, Bert *et al.*, 2005, de Kloet E and de Kloet SR, 2004, Rahaus and Wolff, 2003).

This study aimed to understand the related statistics of APV and PBFDV in parrots such as the positive percentage and the distribution of various ages, seasons, breeds and areas, in order to obtain more information on the health care of captive parrots in Taiwan today.

Materials and Methods

Experiment Design: Blood samples of parrots with suspected APV or PBFDV infection were collected over a year and the distributions of various ages, seasons, breeds and areas were recorded. After extracting sample DNA and obtaining target products by polymerase chain reaction (PCR) with specific primer pairs, the samples were then sent to the Clinical Molecular and Avian Medicine Laboratory, National Pingtung University of Science and Technology for sequencing and, finally, the above data integrated for statistical analysis. The parrot ages were divided into one-week-old to less than six-months-old, six-months-old to less than one-year-old, one-year-old to less than three-years-old, and three-years-old to less than ten-years-old as four groups; each group contained 180 parrots, making a total of 720 parrots. The seasons were divided into spring (February to April), summer (May to July), fall (August to October) and winter (November to January) as four groups; each group had 180 parrots making a total of 720 parrots. Collection targets were mainly small and medium species such as *Aratinga solstitialis*, *Psittacula krameri*, *Eclectus roratus* and Lorikeets (genus *Eos/Lorius/Trichoglossus*); others were *Psittacus erithacus*, *Melopsittacus undulates*, *Nymphicus hollandicus*, genus *Pionites*, genus *Agapornis* and genus *Amazona*. The areas were divided into household pets and commercial aviaries.

Samples: From January 2019 to December 2019 for 12 months, blood samples of parrots were collected from household pets and commercial aviaries in the northern (Taipei, Keelung, Yilan, Taoyuan, Hsinchu), central (Taichung, Changhua, Nantou) and southern (Chiayi, Tainan, Kaohsiung, Pingtung) Taiwan randomly. Sixty samples were collected every month making a total of 720 samples in a year.

Molecular biology tests: Bymolecular biology testing, the main purpose was to extract sample nucleic acid (DNA) and use specific primer pairs to amplify the specific fragment size of the pathogen. Finally, the pathogen could be distinguished. The detected pathogens were *Avian Polyomavirus* and *Psittacine Beak and Feather Disease Virus*.

Nucleic acid extraction: The collected blood paper samples were shredded and an appropriate amount of sterile water was added, followed by shaking, so that the blood on the paper was dissolved in the sterile water.Then, sterile water mixed with the blood was used as whole blood, in conjunction with the Gene PlusTM Genomic DNA Extraction Miniprep System kit

(VIOGENE, GG2002) steps to purify DNA. The purified blood DNA was to be stored at -20 °C.

Polymerase chain reaction assay: The purified DNA was subjected to polymerase chain reaction(PCR) with specific primer pairs of a positive control of APV and PBFDV (Table 1). The APV primer pairs were 5'-CAAGCATATGTCCCTTTATCCC-3' and 5'-CTGTTT AAGGCCTTCCAAGATG-3' which were used for the detection of the t/T antigen coding region of APV. The expected PCR product was 310 bp (Phalen, *et al.*, 1991); the PBFDV primer pairs were 5'-AACCCTACAGAC GGCGAG-3 ' and 5'-GTCACAGTCCTCCTTGTACC-3' which were used to detect the PBFDV genome. Primers were designed from the ORF V1 gene sequence. The expected PCR product was 717 bp. (Ypelaar, *et al.*, 1999).

Purification of PCR products: After confirming the PCR product fragments using 1.5% Agarose gel, the Agarose gel of the target fragment was cut to an appropriate size for purifying target DNA using FavorPrep™ GEL / PCR Purification Mini Kit (FAVOGEN, FAGCK001-1). The purified target DNA had to be stored at -20°C until use.

Table 1   Specific primer pairs of APV and PBFDV.

Name	Sequence (5′–3′)	Region	Position	Size	References
APV	CAAGCATATGTCCCTTTATCCC CTGTTTAAGGCCTTCCAAGATG	t/T antigen	4303-4324 4612-4591	310bp	(Phalen, *et al.*, 1991)
PBFDV	AACCCTACAGACGGCGAG GTCACAGTCCTCCTTGTACC	ORF V1	182-199 898-879	717bp	(Ypelaar, *et al.*, 1999)

Recombinant plastids and colonies: The purified target DNA was prepared using the RBC TA Cloning Vector Kit (RBC, RC001) to make DNA recombinant plastids. After transforming in DH5α (*Escherichia coli, E. coli* DH5alpha, PTA-7297) competent cells, a single bacterial colony was obtained and scaled up. Using an RBC vector-specific primer pair to confirm the target fragment, the bacteria was then mixed with 30% glycerin and the mixture frozen at -80°C. Purifying the recombinant plastids was done with FavorPrep™ Plasmid Extraction Mini Kit (FAVORGEN, FAPDE 100) and the pure recombinant plastid DNA obtained.

Sequencing: The pure recombinant plastid DNA was sent to Purigo Biotech, Inc. for sequencing. The primers used for sequencing were M13F(-40): 5'-CAGGGTTTTCCCAGTCACGAC-3' and M13R(-48): 5'-AGCGGATAACAATTTCACACAGG-3' (Table 2). After sequencing, the sequences were compared with data from the NCBI(National Center for Biotechnology Information, U.S. National Library of Medicine) GenBank.

Table 2   The sequencing primers used in this study.

| Primer name	Sequence (5′-3′)	Source
M13F(-40)	CAG GGT TTT CCC AGT CAC GAC	Purigo Biotech, Inc.
M13R(-48)	AGC GGA TAA CAA TTT CAC ACA GG	Purigo Biotech, Inc.

Results

Results of PCR: The Agarose gel electrophoresis showed that M was the Marker, which was the exact position of the positive control for determining the results of electrophoresis; the positive control of APV was located at 310bp and the positive control of PBFDV was located at 717bp (Figure 1).

Sequence analysis: The sequences were amplified by PCR which, after sequencing, were compared with

data from the NCBI GenBank to determine that they were specific nucleic acid fragments of APV and PBFDV and that there was no genetic variation.

Total positive percentage: A total of 62 of the 720 parrots (8.6%) were APV positive and a total of 204 of the 720 parrots (28.3%) were PBFDV positive. A total of 87 of the 720 parrots (12%) were APV/PBFDV co-infections (Table 3).
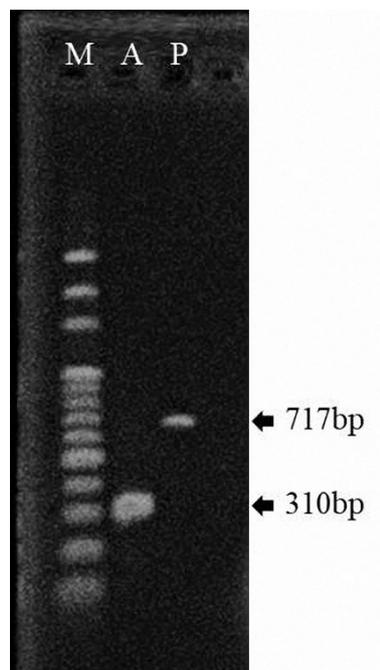
Figure 1   Agarose gel electrophoresis showing the two PCR products of APV t/T antigen and PBFDV ORF V1 gene from the positive control. Lane 1 (M): DNA Molecular Weight Marker (GeneDireX, Inc., Taiwan), lane 2(A): 310bp from the positive control of APV, lane 3(P): 717bp from the positive control of PBFDV respectively.

Table 3   Various positive percentages of APV and PBFDV in parrots.

Items	Sub-Items	Kinds of Virus and Various positive percentages				
		APV	PBFD	APV*	PBFD*	APV/PBFDV Co-infection*
Total		8.6% (62/720)	28.3% (204/720)	-	-	12% (32/266)
Age	1 week~<6 months	14.4% (26/180)	71.1% (128/180)	42% (26/62)	63% (128/204)	-
	6 months~<1 yr	10% (18/180)	26.7% (48/180)	29% (18/62)	23% (48/204)	-
	1~<3yrs	6.7% (12/180)	10% (18/180)	19% (12/62)	9% (18/204)	-
	3~<10yrs	3.3% (6/180)	5.6% (10/180)	10% (6/62)	5% (10/204)	-
Season	Spring	13.3% (24/180)	44.4% (80/180)	39% (24/62)	39% (80/204)	-
	Summer	4.4% (8/180)	15.6% (28/180)	13% (8/62)	14% (28/204)	-
	Autumn	13.9% (25/180)	38.9% (70/180)	40% (25/62)	34% (70/204)	-
	Winter	2.8% (5/180)	14.4% (26/180)	8% (5/62)	13% (26/204)	-
Area	Household pets	8.3% (10/120)	21.7% (26/120)	16% (10/62)	13% (26/204)	-
	Commercial aviaries	8.7% (52/600)	29.7% (178/600)	84% (52/62)	87% (178/204)	-
Breed	*Aratinga solstitialis*	7.5% (16/214)	28% (60/214)	26% (16/62)	29% (60/204)	-
	Psittacula krameri	14.1% (22/156)	48.7% (76/156)	35% (22/62)	37% (76/204)	-
	Eclectus roratus	6.3% (6/96)	22.9% (22/96)	10% (6/62)	11% (22/204)	-
	Lorikeets	9.8% (8/82)	19.5% (16/82)	13% (8/62)	8% (16/204)	-
	Other	5.8% (10/172)	17.4% (30/172)	16% (10/62)	15% (30/204)	-

Proportion of positive samples.

Age distribution: Of all samples, the positive percentages of APV from the four groups which were young to old in order were 14.4% (26/180), 10% (18/180), 6.7% (12/180) and 3.3% (6/180), while of PBFDV they were 71.1%(128/180), 26.7%(48/180), 10%(18/180) and 5.6%(10/180). In the positive samples, the proportion of APV from the four groups which were young to old in order were 42%(26/62), 29%(18/62), 19%(12/62) and 10%(6/62), while of PBFDV they were 63%(128/204), 23%(48/204), 9%(18/204) and 5%(10/204). The proportion of APV positive and PBFDV positive in all samples or positive

samples were both highest in psittacine chicks that were under six-months-old and this decreased with age (Table 3).

Season distribution: Of all samples, the positive percentages of APV from spring to winter in order were 13.3%(24/180), 4.4%(8/180), 13.9%(25/180) and 2.8%(5/180), while of PBFDV they were 44.4%(80/180), 15.6%(28/180), 38.9%(70/180) and 14.4%(26/180). In the positive samples, the proportion of APV positive from spring to winter in order were 39%(24/62), 13%(8/62), 40%(25/62) and 8%(5/62), while of PBFDV positive they were 39%(80/204), 14%(28/204), 34%(70/204) and 13%(26/204). The highest proportions of APV positive and PBFDV positive in all samples or positive samples were both in spring and fall (Table 3).

Area distribution: A total of 52 of 600 parrots (8.7%) of commercial aviaries and 10 of 120 parrots (8.3%) that were household pets were APV positive. A total of 178 of 600 parrots (29.7%) of commercial aviaries and 26 of 120 parrots (21.7%) that were household pets were PBFDV positive. In positive samples, the proportion of APV was 84% (52/62) of commercial aviaries and 16% (10/62) of household pets; the proportion of PBFDV was 87% (178/204) of commercial aviaries and 13% (26/204) of household pets. Of all or positive samples, the highest was in commercial aviaries (Table 3).

Breed distribution: Of all samples, the positive percentages of APV and PBFDV were 7.5% (16/214) and 28% (60/214) in *Aratinga solstitialis*, 14.1% (22/156) and 48.7% (76/156) in *Psittacula krameri*, 6.3% (6/96) and 22.9% (22/96) in *Eclectus roratus*, 9.8% (8/82) and 19.5% (16/82) in Lorikeets (genus *Eos/Lorius/Trichoglossus*), 5.8% and 17.4% in other breeds, showing that the highest for APV and PBFDV positive were in *Psittacula krameri*; the second-highest for APV and PBFDV positive were in Lorikeets and *Aratinga solstitialis* respectively. In positive samples, the proportions of APV and PBFDV were 26% (16/62) and 29% (60/204) in *Aratinga solstitialis*, 35% (22/62) and 37% (76/204) in *Psittacula krameri*, 10% (6/62) and 11% (22/204) in *Eclectus roratus*, 13% (8/62) and 8% (16/204) in Lorikeets and 16% (10/62) and 15% (30/204) in other breeds. The highest and second-highest for APV and PBFDV in positive samples were both in *Psittacula krameri* and *Aratinga solstitialis* respectively (Table 3).

Discussion

The positive percentage of PBFDV in this study is 28.3%, which is higher than that of the United States, Italy and Costa Rica but lower than that of Germany. The positive percentage of APV in this study is 8.6%, which is higher than that of Italy and Costa Rica. In terms of APV/PBFDV co-infection, 12% of this study were higher than Costa Rica. This may be related to environment, climate, feeding methods or bird species. The main object of this study is small and medium-sized parrots, which is different from the study of Costa Rica which was mainly of medium and large-sized parrots such as *Ara macao* and *Amazona*

autumnalis (Dolz *et al.*, 2013, Bert *et al.*, 2005, De Kloet E and De Kloet SR, 2004, Rahaus and Wolff, 2003).

Compared with the previous two studies of Taiwan, the PBFDV positive percentage was the highest in 2002-2005 at 41.2% (Hsu *et al.*, 2006) but it dropped to 8.59% from 2010 to 2014 (Thongchan *et al.*, 2015) and rose again in 2019 to 28.3%. The APV positive percentage decreased significantly from 15.2% (Hsu *et al.*, 2006) and 16.56% (Thongchan *et al.*, 2015) to 8.6%, almost by half, which might have been caused by climate change or bird migration. However, the positive percentage of APV/PBFDV co-infection in this study is 12%, compared with previous studies which were 10.3% (Hsu *et al.*, 2006) and 11.04% (Thongchan *et al.*, 2015), and there is no significant difference. The main species most susceptible to being APV and PBFDV positive in this study were *Psittacula krameri*, Lorikeets, *Aratinga solstitialis*, *Eclectus roratus*, especially in chicks that were under six-months-old, which is the same as the susceptible species described in the literature (Thongchan *et al.*, 2015, Johne and Muller, 2007, Hsu *et al.*, 2006, Stoll *et al.*, 1993).

Among them, the positive percentage of APV and PBFDV was the highest in the *Psittacula krameri*, which could be related to the higher proportion of the rising numbers in Taiwan and the crowded feeding environment. In Taiwan, the breeding season for most parrots is mainly from late winter to spring, while lorikeets mainly breed in the fall. This shows a consistency with the results of this study with the greatest APV and PBFDV infection mainly in spring and fall. The two viruses are easily spread during the breeding season when parrots are exposed to frequent contact, especially when young birds have poor immunity and are infected with viruses from the droppings of infected adult birds and the dust of feathers or feces (Ritchie *et al.*, 1991, Gerlach, 1986). And this is the reason why a higher proportion of APV and PBFDV infection is from commercial aviaries rather than that from household pets.

For feeding management, the birdhouse should be kept clean and attention paid to ventilation. If the parrots raised are found to be sick, the sick birds must be isolated and the environment must be disinfected.

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